5-Plex STAT Panel – Phosphoprotein

Cat. # 48-610

MILLIPLEX® MAP

5-Plex STAT Panel – Phosphoprotein
(STAT1, STAT2, STAT3, STAT5A/B, and STAT6)

#48-610

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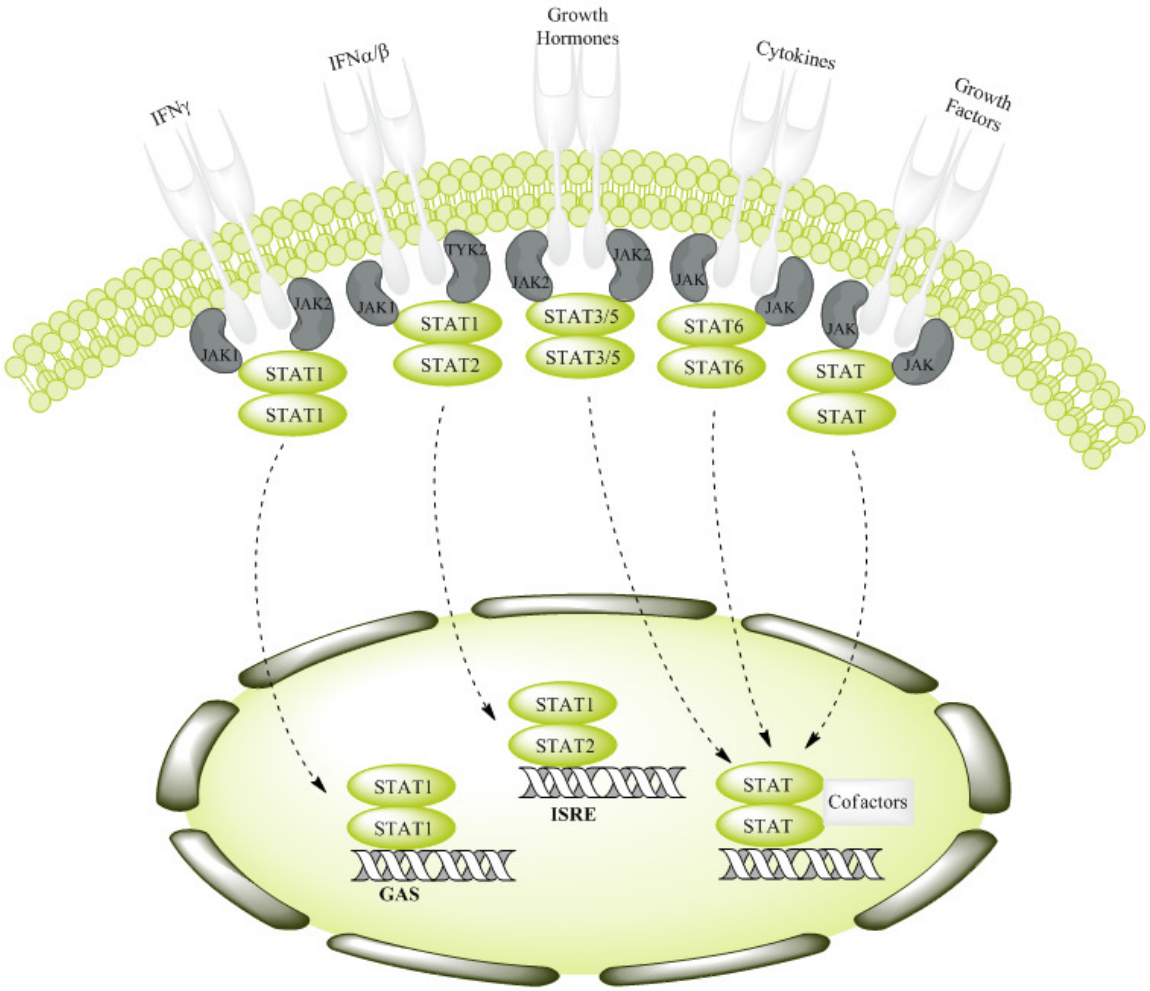
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

STATs (Signal Transducers and Activators of Transcription) are utilized by receptors for a wide variety of ligands including cytokines, hormones, growth factors and neurotransmitters. The phosphorylation of STAT proteins at conserved tyrosine residues activates SH2-mediated dimerization followed rapidly by nuclear translocation. STAT dimers bind to IRE (interferon response element) and GAS (gamma interferon-activated sequence) DNA elements, resulting in the transcriptional regulation of downstream genes. STAT pathways play important role in oncogenesis, tumor progression, angiogenesis, cell motility, immune responses and stem cell differentiation.

STAT Pathways



The MILLIPLEX™ MAP 5-plex STAT Panel, phosphoprotein, is used to detect changes in phosphorylated STAT1 (Tyr701), STAT2 (Tyr690), STAT3 (Tyr705), STAT5A/B (Tyr694/Tyr699), and STAT6 (Tyr641) in cell lysates using the Luminex® system. The MILLIPLEX assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for one 96 well plate assay.

PRINCIPLE

MILLIPLEX MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

This kit is for research purposes only.

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation ("Luminex"), you, the customer, acquire the right under Luminex's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex's laser based fluorescent analytical test instrumentation marketed under the name of Luminex100, 200, HTS.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
MILLIPLEX MAP 5-plex STAT Panel-Phosphoprotein, Beads (20x)	42-610K	131 µL	1 tube
MILLIPLEX MAP 5-plex STAT Panel-Phosphoprotein, Biotin (20x) (Detection Antibody)	44-610K	131 µL	1 tube
MILLIPLEX MAP Lysis Buffer	43-040	55 mL	1 bottle
MILLIPLEX MAP Assay Buffer 2	43-041	55 mL	1 bottle
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated	47-205	----------	1 vial
MILLIPLEX MAP HeLa Cell Lysate: IFNα	47-226	----------	1 vial
MILLIPLEX MAP Daudi Cell Lysate: IL-4	47-217	----------	1 vial
MILLIPLEX MAP Streptavidin-Phycoerythrin	45-001D	115 µL	1 tube
MILLIPLEX MAP Amplification Buffer (1X)	43-024A	3 mL	1 bottle
MILLIPLEX MAP 96-well Filter Plate	MX-PLATE	----------	1 plate
Empty mixing vials	----------	----------	3 vials

Protein	Bead Set
STAT1	24
STAT2	67
STAT3	14
STAT5	35
STAT6	10

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Protease inhibitors (recommend Millipore Catalog #20-201 or similar product)
- Coomassie or BCA-based total protein assay or an assay normalization control, such as the GAPDH MAPmates (catalog #46-667)

Instrumentation / Materials

- Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
- Multichannel Pipettes capable of delivering 25 µL to 200 µL
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Rubber Bands
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator
- Titer Plate Shaker
- Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
- Luminex 100™ IS, 200™, or HTS by Luminex Corporation
- Plate Stand (Millipore Catalog # MX-STAND)
- Filter devices for clearing lysates
  - 2 mL or greater, Millipore Catalog # SLPBDZ5NZ
  - 0.5 – 2 mL, Millipore Catalog # UFC 0DV 25
  - Less than 0.5 mL, Millipore Catalog # UFC30DV00
  - For 96 well plates, Millipore Catalog # MSNBVN1210

SAFETY PRECAUTIONS

- All tissue components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with an opaque plate lid or aluminum foil during all incubation steps.
- Mix only the required amount of beads and biotin-labeled detection antibody prior to assay setup. Discard any unused diluted premixed beads and biotin-labeled detection antibodies.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a plate stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the appropriate Assay Buffer provided.
- After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µL of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Considerations for Cell Stimulation.

- Treating cells with growth factors (ex. insulin) induce a multitude of signaling cascades. The duration of stimulation in addition to the concentration of the respective factor/compound should be considered since they influence the degree of phosphorylation of any given analyte.
- Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
- Cell lines will differ in the robustness of their signaling response for any given stimulation.
- The suggested working range of protein concentration for the assay is 1 to 25 µg of total protein/well (25 µL/well at 40 to 1000 µg/mL). A total protein amount of 10 µg/ well is generally a good starting point for lysates for which target protein expression levels are unknown.

B. Preparation of cell lysates

MILLIPLEX MAP Lysis Buffer is supplied as **1X** stock solution. The lysis buffer contains phosphatase inhibitors *including* 1 mM sodium orthovanadate (Na_3VO_4) but does ***NOT*** contain protease inhibitors. It is recommended that protease inhibitors (Millipore catalog #20-201 or a similar product) be added immediately before use.

<u>Suggested cell lysis protocol for adherent cells</u>

- After treatments, wash cells with ice cold Phosphate Buffered Saline (PBS) and drain off PBS.
- Add ice cold **1X** MILLIPLEX MAP Lysis Buffer with freshly added protease inhibitors to cells (0.6 mL per 150 mm dish, 0.3 mL per 100 mm dish, or 0.1 mL per well of 24-well plate).
- Scrape adherent cells off the dish with a cell scraper. Transfer the cell suspension into a centrifuge tube and gently rock for 10-15 minutes at 4°C.
- Remove particulate matter by filtration.
  Suggested Millipore filters:
  - 2 mL or greater, Millipore Catalog # SLHVX13NL
  - 0.5 – 2 mL, Millipore Catalog # UFC40DV25
  - Less than 0.5 mL, Millipore Catalog # UFC30DV25
  - For 96 well plates, Millipore Catalog # MSBVN1210
- Aliquot and store the lysate at -70°C. The lysate should be stable for several months.
- It is recommended that the lysate be diluted at least 1:10 with PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmates (catalog #46-667), is used.

<u>Suggested cell lysis protocol for non-adherent cells</u>

- Pellet the cells by centrifugation (500 – 1000 x g) in a tabletop centrifuge for 5 minutes.
- Wash the cells in ice cold TBS.

- Add ice cold **1X** MILLIPLEX MAP Lysis Buffer containing freshly prepared protease inhibitors to cells (1 mL per 1 x 10^7 cells).
- Gently rock the lysate for 10-15 minutes at 4°C.
- Remove particulate matter by filtration (See above). Aliquot and store the lysate at -70°C. The lysate should be stable for several months.
- It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmates (catalog #46-667), is used.

Cell lysis protocol for cells in sterile 96-well tissue culture plates

Adherent or non-adherent cells seeded or grown in sterile 96-well tissue culture grade plates (See supplemental protocols) can be washed, treated, and lysed in the same plate, but need to be filtered in a separate 96-well filter plate. Wash the cells by centrifugation in a microplate carrier 2 minutes at 500 x g.

- Remove the supernatant via aspiration and add 100 µL of ice cold PBS.
- Centrifuge and remove supernatant via aspiration.
- Add 30-50 µL/well of ice cold 1X MILLIPLEX MAP Lysis Buffer containing freshly prepared protease inhibitors.
- Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C.
- Transfer the lysate to a 96-well filter plate that has been pre-wetted with 1X lysis buffer.
- Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
- Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
- Store the filtered lysate at -70°C until ready for use.
- It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmates (catalog #46-667), is used.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of antibody Multi-pathway phosphoprotein beads

MILLIPLEX MAP capture beads are provided as a **20X** stock solution and should be protected from light.

- Sonicate **20X** stock capture beads for 15 seconds, then vortex for 30 seconds.
- Dilute the beads to **1X** by combining 0.125 mL beads with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty mixing vial provided.
- Vortex the **1X** capture beads for 15 seconds.
- For use, transfer 1X capture beads with a pipette, do not pour from mixing vial.
- The addition of total or pTyr MAPmate pairs to this panel is not recommended due to bead or detector cross-reactivity

B. Preparation of Biotin-Labeled Detection Antibody and Streptavidin-PE

MILLIPLEX MAP Detection Antibody is provided as a **20X** stock solution.

- Vortex the **20X** Detection Antibody stock for 10 seconds, it may be necessary to centrifuge briefly after vortexing for complete recovery of contents.
- Dilute the Detection Antibody to **1X** by combining 0.125 mL of Detection Antibody with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty bead-mixing vial provided.
- Vortex the MILLIPLEX MAP Streptavidin-Phycoerythrin 1:25 (SAPE) for 10 seconds.
- Dilute SAPE 1:25 by combining 0.1ml of Streptavidin-Phycoerythrin with 2.4 mL of Milliplex MAP Cell Signaling Assay Buffer 2. Use the empty mixing vial provided.
- Transfer 1X biotinylated detection antibody and SAPE with a pipette do not pour from mixing vial.

C. Multiplexing additional MILLIPLEX MAP Cell Signaling MAPmates with the 5-plex STAT Panel, phosphoprotein.

Additional Cell Signaling Phospho-MAPmates may be combined with this kit, up to a maximum of 7 additional MAPmates.

Please note that Total or pTyr MAPmate pairs should not be multiplexed with the 5-plex STAT Panel, phosphoprotein.

- For each additional MAPmate, sonicate **20X** stock capture beads for 15 seconds, then vortex for 30 seconds.
- Add 125 µL 5-plex STAT Panel beads to the mixing vial
- For each additional MAPmate, add 125 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 2.5 mL with Assay Buffer 2. Vortex the mixed beads well.
- Use the same preparation volumes for the Detection Antibody

Example 1: When using 2 additional MAPmates, add 125 µL 5-plex STAT Panel Beads/ Detection Antibody and 125 µL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 2.125 mL Assay Buffer 2, for a final volume of 2.5 mL.

Example 2: When using 5 additional MAPmates, add 125 µL 5-plex STAT Panel Beads/ Detection Antibody and 125 µL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 1.75 mL Assay Buffer 2, for a final volume of 2.5 mL.

D. Preparation of lyophilized MILLIPLEX MAP Cell Lysates (Catalog # 47-205, 47-226 and 47-217).

MILLIPLEX MAP HeLa Cell Lysate: Unstimulated (#47-205) is provided as a lyophilized stock of cell lysate prepared from unstimulated HeLa cells and is used as a negative control. MILLIPLEX MAP HeLa Cell Lysate: IFNα (#47-226) is provided as a lyophilized stock of cell lysate prepared from HeLa cells stimulated with 10,000U/mL IFNα (15 min). MILLIPLEX MAP Daudi Cell Lysate: IL-4 (#47-217) is provided as a lyophilized stock of cell lysate prepared from Daudi cells stimulated with 50 ng/mL IL-4 (10 min). Each of the cell lysates were prepared in MILLIPLEX MAP Lysis Buffer containing protease inhibitors and lyophilized for stability. The lysates can be used as positive and negative control samples or alternatively, to create calibration curves for relative quantification of different phosphoprotein analytes.

MILLIPLEX MAP Cell Lysates as a positive and negative control

- Reconstitute each of the lyophilized cell lysates in 100 µL of ultrapure water, for each vial this will yield 100 µL of lysate at 2 mg/mL total protein.
- Gently vortex and incubate the reconstituted lysates for 5 min at RT (store on ice).
- Pipette 150 µL of MILLIPLEX MAP Assay Buffer 2 to each cell lysate vial. The cell lysate is now prepared for use in the MILLIPLEX MAP 5-plex STAT Panel Cell Signaling Assay.
- If desired, unused lysate may be stored in its original container at -80°C for up to one month.

IMMUNOASSAY PROTOCOL

1. Dilute filtered lysates at least 1:1 in MILLIPLEX MAP Assay Buffer 2. The suggested working range of protein concentration for the assay is 1 to 25 µg of total protein/well (25 µL/well at 40 to 1,000 µg/mL).
2. Pre-wet filter plate with 25 µL/well of MILLIPLEX MAP Assay Buffer 2. Remove by vacuum filtration by placing the filter plate over a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
3. Vortex the **1X** bead suspension for 10 seconds. Add 25 µL of 1X bead suspension to each well.
4. Add 25 µL of diluted cell lysate (or reconstituted HeLa or HepG2 Cell Lysate Control) to each well and incubate overnight (16-20 hours) at 4°C (2 hours RT is not recommended) on a plate shaker (600-800rpm) protected from light.
5. Remove the lysate by vacuum filtration.
6. Add 100 µL/well of MILLIPLEX MAP Assay Buffer 2. Remove buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel. Repeat this step again for a total of two washes.
7. Add 25 µL/well of **1X** MILLIPLEX MAP Detection Antibody.
8. Incubate on a plate shaker for 1 hour at room temperature, protected from light.
9. Remove Detection Antibody by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
10. Add 25 µL of 1X MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE).
11. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
12. **DO NOT REMOVE** SAPE. Add 25 µL of MILLIPLEX MAP Amplification Buffer to each well.

Add 25 µL Assay Buffer per well

↓ Remove buffer by vacuum.

- Add 25 µL 1X beads to wells
- Add 25 µL diluted cell lysate to appropriate wells

↓ Incubate overnight at 4°C with shaking; dark

Wash 2X with 100 µL Assay Buffer. Add 25 µL 1X Detection Antibody.

↓ Incubate 1 hr at RT with shaking; dark

Remove Detection Antibody and add 25 µL 1X Streptavidin-PE (SAPE)

↓ Incubate 15 min at RT with shaking; dark

DO NOT REMOVE SAPE and add 25 µL Amplification buffer

13. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
14. Remove MILLIPLEX MAP SAPE /Amplification buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
15. Resuspend beads in 150 µL of MILLIPLEX MAP Assay Buffer 2, and mix on plate shaker for 5 minutes.
16. Analyze using the Luminex® system.

Incubate 15 min at RT with shaking; dark

Remove Streptavidin-PE/ Amplification buffer and resuspend beads in 150 µL assay buffer. Read results using appropriate Luminex® instrument.

INSTRUMENT SETTINGS

These specifications are for Luminex100/200 instruments. Luminex instruments with other software (e.g. MasterPlex, StarStation, LiquiChip, Bio-Plex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications from the vendors.

Events	50 per bead	
Sample Size	100 µL	
Gate Settings	8,000 to 15,000	
Reporter Gain	Default (Low PMT)	
Time Out	60 seconds	
Bead Set		
	STAT6	10
	STAT3	14
	STAT1	24
	STAT5	35
	STAT2	67

SUPPLEMENTAL PROTOCOLS

A. Analysis of viscous cell lysates

Some cell lysates may not flow through the filter plate efficiently due to high viscosity or the formation of particulate matter from long-term storage. For these samples, the initial capture and wash steps can be done in microcentrifuge tubes. The beads are then transferred into 96-well filter plates for the rest of the assay.

- Add 25 µL/assay point of 1X beads to a 500 µL centrifuge tube.
- Next, add lysate diluted in MILLIPLEX MAP Assay Buffer 2 to a final volume of 100 µL or higher.
- Vortex the mixture at high speed for 15 seconds then sonicate for an additional 15 seconds.
- Rotate the mixture overnight at 4°C, protected from light.
- Centrifuge the beads for 1 min at 2,000 x g and carefully remove the supernatant to minimize bead loss.
- Resuspend the pelleted beads in 25 µL/assay point of MILLIPLEX MAP Assay Buffer 2.
- Transfer 25 µL of the bead mixture to pre-wet filter plate wells and proceed to step 4 of the Immunoassay protocol.

REPLACEMENT REAGENTS

MILLIPLEX MAP 5-plex STAT Panel-Phosphoprotein, Beads (20x)	42-610K
MILLIPLEX MAP 5-plex STAT Panel-Phosphoprotein, Biotin (20X) (Detection Antibody)	44-610K
MILLIPLEX MAP Lysis Buffer	43-040
MILLIPLEX MAP Assay Buffer 2	43-041
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated	47-205
MILLIPLEX MAP HeLa Cell Lysate: IFNα	47-226
MILLIPLEX MAP Daudi Cell Lysate: IL-4	47-217
MILLIPLEX MAP Streptavidin-Phycoerythrin	45-001D
MILLIPLEX MAP Amplification Buffer (1X)	43-024A
MILLIPLEX MAP 96-well Filter Plate	MX-PLATE

REPRESENTATIVE DATA

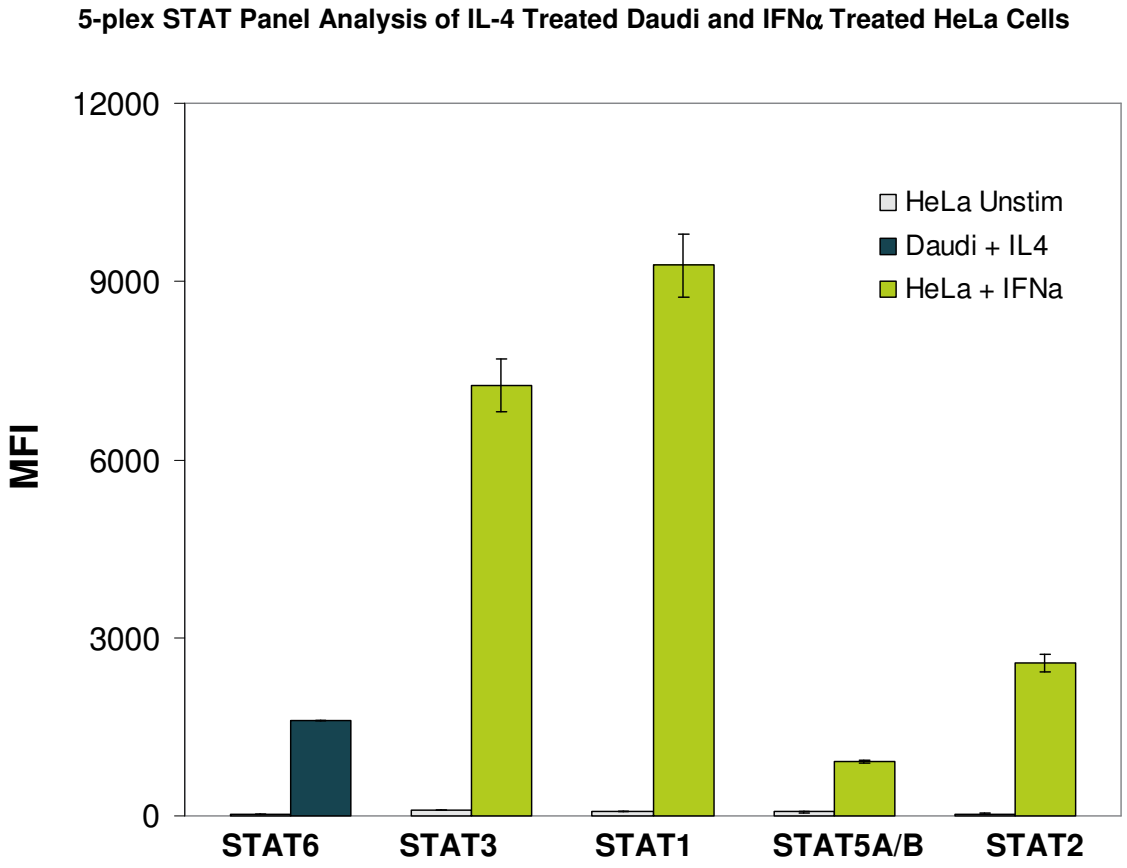


Figure 1. Multiplex analysis of Daudi and HeLa cells treated with IL-4 or IFNα. Unstimulated HeLa cells, HeLa cells stimulated with 10,000U/mL of IFNα (15 min) or Daudi cells stimulated with 50 ng/mL IL-4 (10 min) were assayed. The cells were lysed in MILLIPLEX MAP Lysis Buffer containing protease inhibitors. 20 µg total protein of each lysate diluted in MILLIPLEX MAP Assay Buffer 2 were analyzed according the Assay protocol (lysate incubation at 4ºC overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate wells.

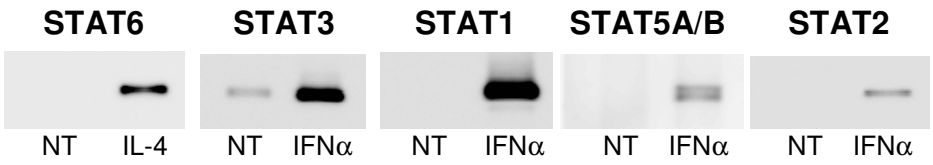


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed STAT analytes. 50 µg of lysates (nontreated, NT, or treated with 50 ng/mL IL-4 or 10,000U/mL IFNα were mixed with capture antibodies to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled phospho-specific detection antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescence.

TROUBLESHOOTING GUIDE

Situation	Possible Problem	Solution
Data acquisition time exceeds 30 seconds per well and/or "Sample Empty" occurs	**Mechanical** 1. Needle height is not correct (common problem due to variation in well depth between plate types). 2. Sample needle is clogged. 3. Air in the system. 4. Low pressure in the system. **Sample Related** 1. Lysate concentration too high. 2. Particulate matter in lysate. 3. No buffer in well.	**Mechanical** 1. Adjust needle height using 2 disks using "options; XY setup function". 2. Remove needle (see user's manual) and sonicate it to remove obstruction. 3. Perform alcohol flush and then wash with alcohol; followed by a wash with sheath fluid and a prime. 4. Tighten tops on the ***sheath fluid*** container. 5. Loosen the top on the ***waste*** container. **Sample Related** 1. Refer to lysate preparation protocol. 2. Centrifuge or filter lysate to remove particulate matter.
Readings are lower than expected	1. Gate is not set properly. 2. Calibration is not correct. 3. Cells responded poorly to stimulation.	1. Right click within the "doublet discriminator" and create gate. Move lines to 8,000 and 13,500 2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Check stimulation conditions.

Bead pattern is diffuse and missing the bead target (white oval)	1. Precipitate buildup in system. 2. Calibration is not correct. 3. Incompatible buffer used to resuspend beads for Luminex® analysis.	1. Drain the system, followed by a backflush, proceed with solution for air in the system. 2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Vacuum plate and resuspend beads in MILLIPLEX MAP Assay Buffer 2.
Applying vacuum to filter plate does not remove liquid from wells	1. Wells not in use are empty. 2. Particulate matter in lysate.	1. Place tape over the top of empty wells that are not in use. 2. Pre-filter lysate before use.
Aggregation of beads during analysis	1. Particulate matter in lysate. 2. Incompatible lysis buffer. 3. Beads not sonicated for step 3 of main protocol.	1. Pre-filter the lysate. 2. Use recommended lysis buffer. 3. Sonicate ***resuspended*** 1X beads prior to adding to filter plate.
Liquid wicking out from 96-well filter plate	1. Plate was not blotted on paper towel prior to adding next reagent. 2. The bottom of the plate is in contact with absorbent material such as paper towel or bench paper.	1. After vacuum step, gently blot the bottom of the plate using a paper towel to remove excess liquid. 2. Place the plate on a flat, non-absorbent surface during loading steps.

ORDERING INFORMATION

To place an order:

FAX: (636) 441-8050

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits

Toll-Free US: (800) MILLIPORE

Mail Orders: Millipore Corp.
Attn: Customer Service
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers in placing an order or obtaining additional information about MILLIPLEX MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at customerserviceEU@Millipore.com.

Conditions of Sale

All products are for research use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for Millipore products may be ordered by fax or phone or through our website at www.millipore.com/techlibrary/index.do

Technical Services

For product technical assistance call or write.

Toll-Free US: (781) 533-8159

Email: techserv_dd@millipore.com

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	HeLa: Unstim negative control											
B	HeLa: Unstim negative control											
C	Daudi: IL-4 positive control											
D	Daudi: IL-4 positive control											
E	HeLa: IFNα positive control											
F	HeLa: IFNα positive control											
G												
H												